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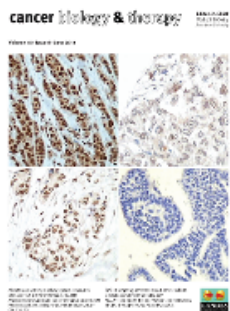
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(Article begins on next page)



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# Melanoma immunotherapy

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**Keywords:** interleukins, interferons, CTLA-4, ipilimumab, PD-1, anti-PD-1, immunoprofiling

**Abbreviations:** FDA, US Food and Drug Administration; IL-2, interleukin-2; HD IL-2, high-dose interleukin 2; NK, natural killer; CR, complete response; SITC, Society for Immunotherapy for Cancer; NRAS, neuroblastoma rat sarcoma viral oncogene; pDNA, plasmid DNA; IFN- $\alpha$ , interferon-alpha; LAK, lymphocyte-activated killer; BCT, biochemotherapy; GM-CSF, granulocyte-macrophage colony-stimulating factor; ACT, adoptive cell therapy; BRAF, v-Raf murine sarcoma viral oncogene homolog B1; TLR, toll-like receptor; CTLA-4, cytotoxic T-lymphocyte antigen 4; irAEs, immune-related adverse effects; gp100, glycoprotein 100 peptide; OS, overall survival; EORTC, European Organisation for Research and Treatment of Cancer; PD-1, programmed death-1; PDL-1, programmed death ligand 1; LAG-3, lymphocyte activation gene-3; TIM-3, T-cell immunoglobulin mucin-3; ALC, absolute lymphocyte count; ICOS, inducible costimulator molecule; IDO, indoleamine 2,3-dioxygenase

Immunotherapy is a cornerstone in the treatment of melanoma, and is intended to modulate the host immunity against the tumor. Immunotherapy can be used in an adjuvant setting, after complete surgical excision in patients with a high risk of disease relapse and as a treatment in advanced (unresectable or metastatic) stages. Development of novel therapeutic approaches and the optimization of existing therapies hold a great promise in the field of melanoma therapy research. Different clinical trials are ongoing, and immunotherapy is showing the ability to confirm durable clinical benefits in selected groups of melanoma patients. The aim of this review is to summarize different types of immunotherapy agents, as well as to discuss different strategies, complementary regimens, and possible biomarkers of response to the treatment.

## Introduction

Melanoma is considered to be one of the best examples of an immunogenic tumor. Different observations have led to this assumption: (1) primary melanomas often exhibit strong lymphocytic infiltration, that could induce partial or complete regression, (2) development of vitiligo is a marker of better prognosis in melanoma patients,<sup>1</sup> and (3) immunotherapies have shown remarkable long-term results.<sup>2,3</sup> The observation that immune response affects tumors biology dates back to the late 1800s, when William B Cooley observed remission of lesions in patients injected with a mixture of dead *Streptococcus pyogenes* and dead *Serratia marcescens* bacteria. Current approaches to cancer immunotherapy include: (1) non-specific stimulation of antitumor immune

response by stimulating endogenous effector cells with cytokines, (2) active immunization, (3) adoptive immunotherapy, and (4) targeting of immune checkpoints or immune regulatory molecules (Fig. 1). Currently approved melanoma therapeutics by the US Food and Drug Administration (FDA) for the treatment of melanoma, are listed in Figure 2. The aim of this review is to summarize different types of immunotherapy agents, as well as to discuss different treatment strategies, complementary regimens, and possible biomarkers of response to the treatment.

## Cytokines

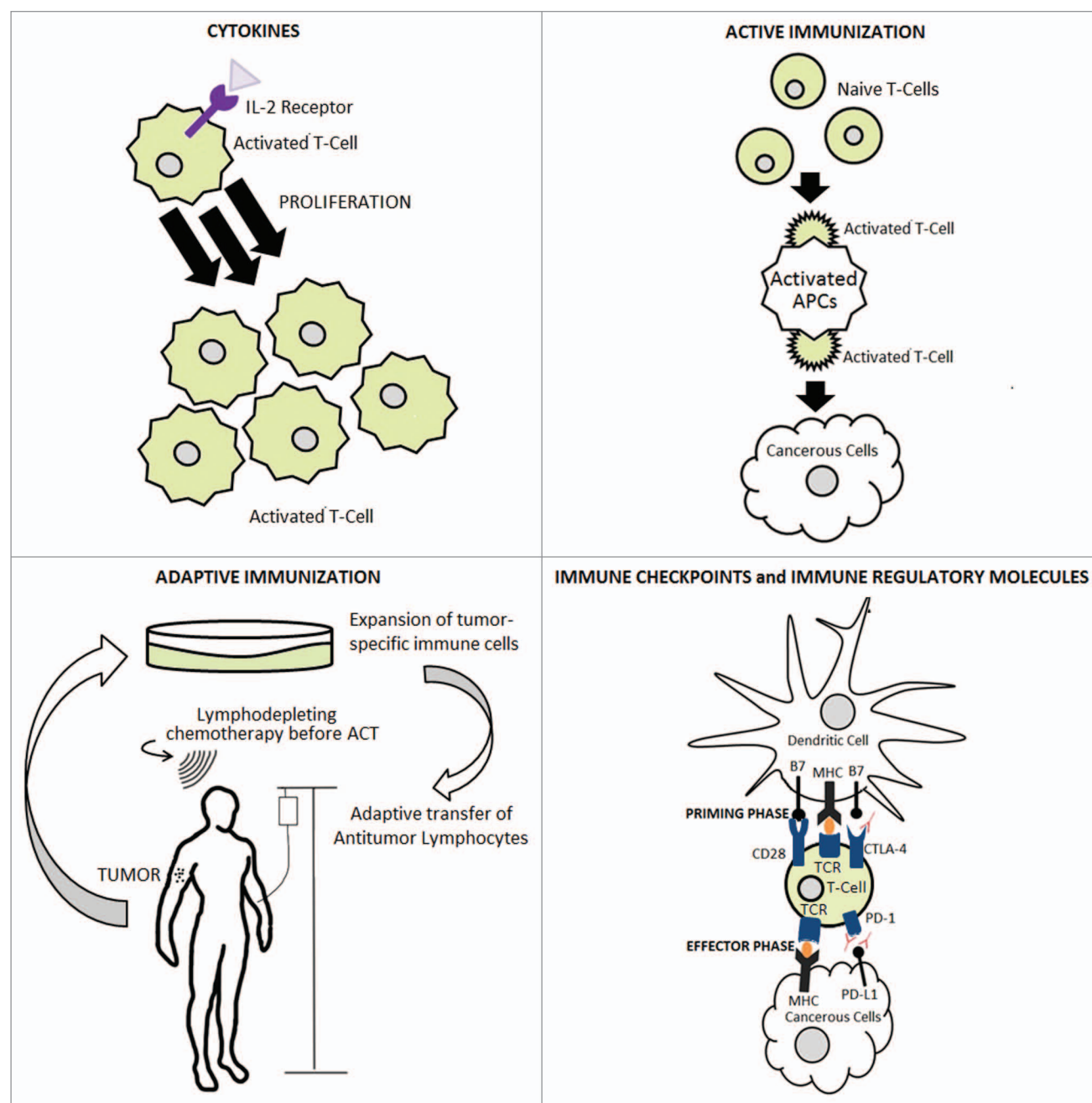
### Interleukin-2 (IL-2)

The first type of immunotherapy approved in the treatment of melanoma was high-dose interleukin 2 (HD IL-2), which provided a “proof-of-principle” for the use of immunotherapy in melanoma.<sup>2</sup> Interleukin-2 plays a central role in the activation and stimulation of T lymphocytes and natural killer (NK) cells. In response to IL-2 stimulation these cells acquire cytolytic properties which is believed to enhance their anti-tumoral properties.<sup>4</sup>

#### IL-2 in advanced melanoma

HD IL-2 is administered ranging from 600 000 to 720 000 IU/kg/i.v. every 8 h for up to 14 consecutive doses over 5 d, followed by a second treatment cycle after 6 to 9 d.<sup>2</sup> Treatment of patients with advanced melanoma with HD IL-2 has demonstrated a complete response (CR) rate of 6% and partial response rate of 10%. Among patients who reach CR, the response can be long-lasting. However, HD IL-2 is associated with significant acute toxicity (severe hypotension, pulmonary edema, systemic edema with significant weight gain and renal insufficiency, rash and fatigue).<sup>5</sup> For this reason HD IL-2 requires the hospitalization and is usually reserved for patients in a good performance status. Alternative regimens have been investigated, but were unable to reach comparable response rates. The Society for Immunotherapy for Cancer (SITC) recommends HD IL-2 as first-line treatment in patients with stage IV BRAF-wild type

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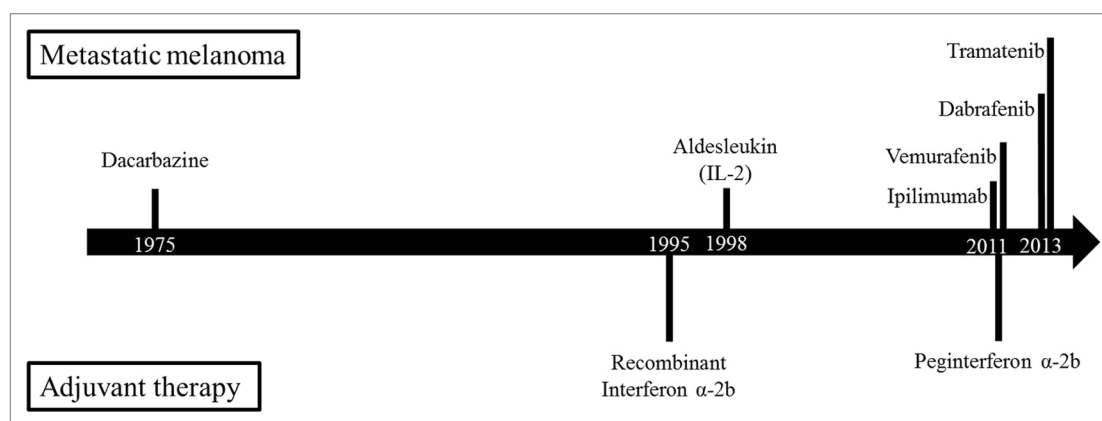
**Figure 1.** Methods in cancer immunotherapy.

melanoma who have a good performance status and no evidence of central nervous system disease.<sup>6</sup> The genetic background of melanoma might also affect response rates for IL-2 treatment. A recent report<sup>7</sup> suggests that neuroblastoma rat sarcoma viral oncogene (NRAS) mutations might predict a better response to IL-2 treatment.

#### *Intralesional IL-2 for in-transit lesions*

Surgical resection is the preferred therapeutic approach for in-transit metastases. However, when surgical excision cannot be pursued, another possible option is the intralesional injection of therapeutics. Injecting IL-2 into a metastatic lesion allows for very high intralesional concentrations without systemic toxicity.

Several small series have reported promising clinical responses in treated lesions.<sup>8-11</sup> Boyd et al. injected 10 million IU of IL-2 in each lesion twice a week in a total of 39 patients and reported complete and partial responses in 51% and 31%, respectively.<sup>8</sup> Radny et al. used single doses from 0.6 to 6 million IU, depending on lesion size and injected 2–3 times weekly in a total of 24 patients. They reported complete and partial responses in 63% and 21%, respectively.<sup>10</sup> A systemic effect of this treatment was suggested, with a higher five-year survival in patients with a complete response compared with patients with a partial response (80% vs 50%).<sup>8</sup> To achieve increased local expression of the cytokine over a prolonged period of time, plasmid DNA (pDNA)



**Figure 2.** Drugs FDA-approved for the treatment of melanoma with approval date.

expression vectors, which deliver the IL-2 gene into tumor cells, are used.<sup>12</sup> The combination of vector delivery via intralesional injection followed by electroporation to facilitate cellular uptake of pDNA is an area of active research.<sup>13</sup>

Several other immunomodulatory gene therapy trials have been conducted; still the most promising results are achieved with administration of pDNA encoding IL-12 along with electroporation. This treatment has also induced responses in untreated lesions, suggesting an induction of systemic response.<sup>14</sup>

#### Interferon-α

Interferons are proteins secreted in order to communicate and trigger immune responses to eradicate pathogens and tumor cells. Interferon-α (IFN-α) directly inhibits the proliferation of tumor cells and increases MHC class I expression, enhancing antigen recognition. Moreover, IFN-α represses oncogene and induces tumor suppressor genes expression, in conjunction with antiangiogenic effects.<sup>15</sup> IFN-α is known to regulate the immune system directly or indirectly by the induction of chemokines secretion. In particular, the JAK-STAT pathway plays a critical role in the signaling events induced by IFN, this pathway activates transcription factors STAT, and the activated STAT translocate to the nucleus followed by binding to IFN-stimulated response elements and modulating transcription of IFN-stimulated genes.<sup>16</sup> For example, STAT1 has been proved to be fundamental for the activation of NK cells in response to IFN in murine models.<sup>17</sup> While STAT1 enhances innate and adaptive immunity, STAT3 appears to play opposite role in tumorigenesis, promoting survival, proliferation, motility, and immune tolerance. Studies in STAT-deficient cells have revealed the existence of reciprocal STAT1:STAT3 regulatory mechanisms, and their relative abundance can be implicated in the biological effects in response to IFN activating stimuli.<sup>18</sup>

#### IFN in adjuvant treatment

Various systemic therapies have been examined for patients with stage II or III melanoma and a high risk of systemic recurrence after surgery excision. The immunotherapeutics approved by the FDA for the adjuvant treatment of melanoma are interferon α-2b and peginterferon α-2b. The rationale for the use of the pegylated form is to reduce its rate of absorption following subcutaneous injection, to reduce renal and cellular clearance,

and to decrease the immunogenicity of the protein. All of these effects tend to enhance the half-life of the pegylated protein. On the other hand, pegylation may interfere with the ability of a protein to bind to its receptor, thereby decreasing its biologic effect. Thus, the true biologic effect of the pegylated protein is determined by the balance of these competing properties of the interferon increases.<sup>19</sup>

Stage III melanoma with macroscopic nodal disease (N1b and N2–N3) is generally considered separately from that with microscopically involved lymph nodes (N1a). The SITC suggests one year of interferon α-2b treatment or enrollment in specific clinical trials, for patients with macroscopic lymph node involvement.<sup>6</sup> On the other hand, in the case of N1a disease, a 1-y course of interferon α-2b, but also no further treatment, or shorter courses of interferon α-2b are suggested. Pegylated interferon α-2b was recommended by a minority of panel members in N1a melanoma with ulcerated primary tumors or for patients unwilling or unable to tolerate the standard regimen of interferon α-2b treatment.<sup>6</sup> Patients with stage II melanoma are considered at high-risk of recurrence if the primary lesion is ulcerated, larger than 4 mm in diameter, or has mitotic rate  $\geq 1$  per mm<sup>2</sup>. Specialists remain divided between recommending interferon α-2b and active surveillance.<sup>6</sup> A recent randomized study showed a better overall survival in patients treated with a 1-y course of interferon α-2b (20 MIU/m<sup>2</sup> intravenously daily 5 d per week for 4 wk, followed by IFN-α-2b 10 MIU/m<sup>2</sup> administered subcutaneously three times per week for 48 wk) compared with a shorter course (20 MIU/m<sup>2</sup> intravenously daily 5 d per week for 4 wk) in patients from stage IIB to stage IIIC.<sup>20</sup> However, most of the patients included had a stage IIIB or IIIC at study entry suggesting once more that 1 y of therapy offers the most benefit to those with macroscopic nodal metastasis and a higher risk of relapse.

#### Biochemotherapy

In the treatment of metastatic melanoma, the combination of chemotherapy with immunotherapy, biochemotherapy (BCT), has been explored in multiple trials. The most used regimen utilizes cisplatin, vinblastine, and dacarbazine with IL-2 and interferon-α-2b. This combination was designed to enhance immune recognition and effector cell activity triggered by IL-2 during antigen release after tumor cell disruption and apoptosis



induced by cytotoxic chemotherapy. Two systemic reviews showed a higher overall response rate with this regimen compared with chemotherapy, but no significant survival advantage.<sup>21,22</sup> A randomized phase III trial compared BTC to interferon- $\alpha$ -2b as adjuvant therapy in 138 patients with stage 3 melanoma and high risk for recurrence, but didn't detect significant differences in overall survival.<sup>23</sup> Another randomized phase III trial compared BTC to interferon- $\alpha$ -2b as adjuvant therapy in 432 patients with stage III melanoma and found that BTC conferred a statistically significant improvement in relapse free survival compared with interferon; but no discernable difference in overall survival with also an higher occurrence of grade IV toxicity.<sup>24</sup>

## Active Immunization

### Vaccines

The aim of tumor vaccination is to induce immune recognition with subsequent specific tumor cell eradication. Multiple approaches have been examined, the simplest strategy has been to administer defined tumor-associated antigens as whole proteins or peptide fragments. Most protein- or peptide-based vaccines have lacked significant immunogenicity and were unable to induce a robust immune response in monotherapy. Therefore, the combination of peptide and/or protein vaccines with nonspecific immunologic adjuvants, such as granulocyte-macrophage colony-stimulating factor (GM-CSF) or HD IL-2, has been investigated. These studies have not demonstrated a consistent benefit in overall survival to date.<sup>25</sup> In an effort to induce a more specific tumor response, the administration of customized vaccines, derived from whole cells or cell lysates of patient's tumor have been tested. However, these have also not shown any clinical benefit in randomized controlled trials. Other approaches that have been evaluated include recombinant vectors, which encode either an entire gene or the antigenic epitope,<sup>26</sup> and dendritic cells pulsed with tumor cell RNA, DNA, or cell lysate.<sup>27</sup>

## Adoptive Immunotherapy

The adoptive cell therapy (ACT) approach utilizes ex vivo cultured autologous lymphocytes. These lymphocytes can be derived from resected metastasis or from peripheral blood. The optimization of the host environment prior to cell transfer appears to be very important for the success of this procedure: high dose chemotherapy is needed to induce lymphodepletion to eliminate immune regulatory elements (both cellular and humoral) that could affect homing and activity of transferred cells.<sup>28</sup> Recent advances in ACT involve the use of autologous engineered T cells that express T-cell receptors specific for various tumor-associated antigens (such as MART-1-TCG genes), or that secrete specific cytokines.

ACT protocols are currently implemented in patients who failed prior systemic treatment for metastatic melanoma. The most common sites to harvest tumor-infiltrating lymphocytes (TILs) are soft tissue and lung metastases. Recently, several

groups published results of phase II clinical trials testing TIL-based ACT.<sup>29-32</sup> The lymphodepleting regimen and the TIL production protocols varied in the different studies. The highest objective response rate (72%) was reported with the use of myeloablative pre-conditioning; whereas the use of CD8<sup>+</sup> T cell-enriched TILs in a non-myeloablative setting was associated with the lower response rates (20%). Interestingly, 19 out of 20 patients in one study<sup>33</sup> and all 5 patients in another study,<sup>34</sup> who completely responded to TIL-based ACT, have no evidence of disease after more than 3 and 2 y, respectively. This shows the great potential of ACT regimens in patients with advanced melanoma stages.

However, the toxicity of preparative chemotherapy mandates to investigate factors that predict treatment success. Combining ACT with other therapy approaches, such as ipilimumab or v-Raf murine sarcoma viral oncogene homolog B1 (BRAF) inhibitors, as well as exploring synergistic effects of cytokines, such as IL15, are future directions, and clinical trials are underway (NCT01701674 and NCT01659151).<sup>35</sup>

## Immune Checkpoints and Immune Modulator Molecules

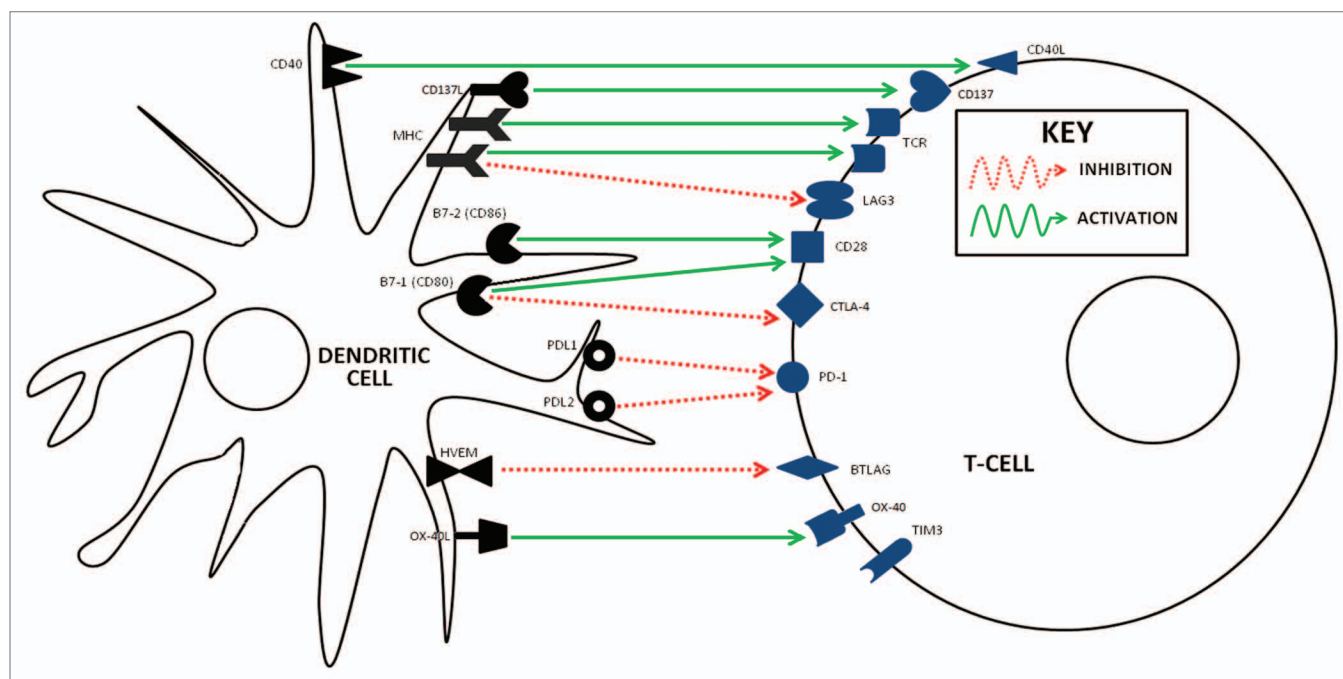
The antitumor T-cell activity depends on the dendritic cell maturation stimulus received from the tumor, and on the successful interaction of T-cell co-stimulatory molecules with surface receptors on dendritic cells (Fig. 3). New immunotherapies target critical regulatory elements of the interaction between T cells, dendritic cells, and tumor cells. These include anti-CTLA-4 monoclonal antibodies such as ipilimumab, toll-like receptor (TLR) agonists, CD40 agonists, anti-PD-1 or PDL-1 antibodies, and others.

### Anti-CTLA-4

The cytotoxic T-lymphocyte antigen 4 (CTLA-4) is a receptor that interacts with CD80 (B7-1) and CD86 (B7-2) and downregulates T-cell response.<sup>36</sup> CD28, although with less affinity than CTLA-4, usually binds to B7-1 and B7-2 leading to T cell activation. CTLA-4 receptors are highly expressed on tumor cell lines and inhibit T-cell responses and "mask" tumors from inducing a host immune response.<sup>37</sup> The excessive CTLA-4 expression on tumor cells represents just one mechanism by which tumors are able to actively suppress and evade T-cell activity. Ipilimumab and tremelimumab (CP-675206) are monoclonal antibodies that block CTLA-4 and allow CD28 to bind to B7-1 receptors, which leads to IL-2 secretion, cytotoxic T-cell activation and proliferation.

### *Anti-CTLA-4 antibodies in metastatic melanoma*

Clinical studies confirm that the CTLA-4 inhibitor ipilimumab can induce durable responses and improve overall survival in patients with advanced melanoma. A phase II study comparing different dose regimens (0.3, 3, or 10 mg/kg IV every 3 wk) established that the best response was obtained with 10 mg/kg. However, at this dose, a significant increase in immune-related adverse effects (irAEs) was observed.<sup>38</sup> In the first phase III trial, ipilimumab +/- glycoprotein 100 peptide (gp100) vaccine was



**Figure 3.** Dendritic cell and T cell interaction.

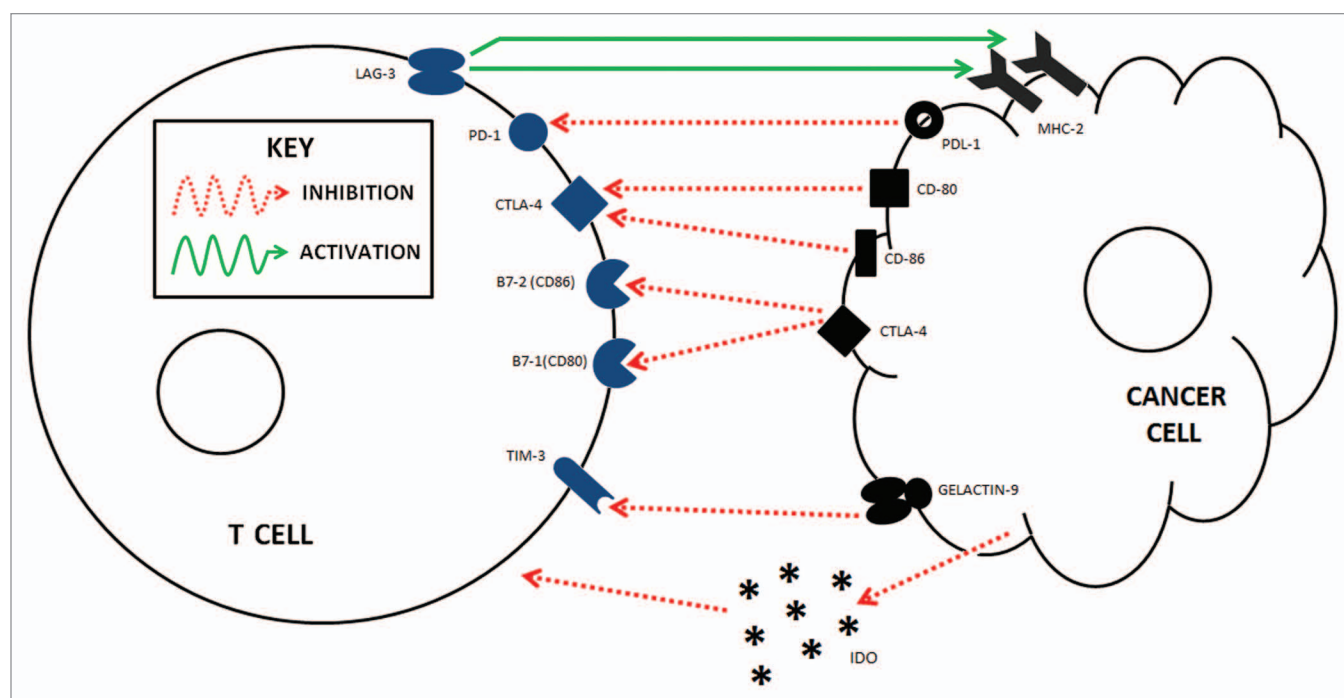
compared with gp100 vaccine monotherapy in patients with unresectable stage III or stage IV melanoma. Ipilimumab in monotherapy improved overall survival (OS) compared with gp100 vaccine monotherapy (10.1 mo vs. 6.4 mo). However, more irAEs were noticed with ipilimumab treatment. The combination of ipilimumab and gp100 did not improve OS as compared with ipilimumab alone.<sup>39</sup> In another randomized phase III trial ipilimumab (10 mg/kg) combined with dacarbazine (850 mg/mq) demonstrated a modest but statistically significant improvement in OS compared with dacarbazine (850 mg/mq) plus placebo (11.2 mo vs. 9.1 mo). A higher incidence of total adverse events was recorded in the ipilimumab–dacarbazine group compared with dacarbazine–placebo including elevation of alanine aminotransferase levels (in 29.1% of patients vs. 4.4%), elevation of aspartate aminotransferase levels (26.7% vs. 3.2%), diarrhea (32.8% vs. 15.9%), pruritus (29.6% vs. 8.8%), and rash (24.7% vs. 6.8%). Grade 3 or 4 adverse events occurred in 56.3% of patients treated with ipilimumab plus dacarbazine, as compared with 27.5% treated with dacarbazine and placebo ( $P < 0.001$ ).<sup>40</sup>

Subsequently, ipilimumab was approved by the FDA in 2011 for the treatment of unresectable or metastatic melanoma. Similar to other types of immunotherapy, ipilimumab demonstrates durable responses in a small subset of patients. Prieto et al. reported on 177 patients treated with ipilimumab in three different trials. 56 patients received ipilimumab with gp100 peptides, 36 patients received ipilimumab with interleukin-2, and 85 patients received ipilimumab with intrapatient dose-escalation and were randomized to receive gp100 peptides. Out of these 177 patients, 9% experienced a complete response and all but one were durable during follow-up ranging from 54 to 99 mo. Responses were most frequently observed in patients who received a combination of HD-IL2 (720000 IU/kg) and ipilimumab.<sup>41</sup>

First results with tremelimumab showed promising activity in phase I and phase II studies. Although, a subsequent phase III randomized trial comparing tremelimumab to chemotherapy (dacarbazine or temozolamide) in 655 patients with advanced melanoma revealed a not significant prolonged overall survival among patients treated with tremelimumab (11.8 mo with tremelimumab vs. 10.7 mo with chemotherapy,  $P = 0.73$ ).<sup>42</sup> The failure of tremelimumab to improve outcome may be attributed to restrictive inclusion criteria of the study: patients with elevated LDH  $>2\times$  upper limit of normal, a known negative prognostic indicator, were excluded from the control cohort, making the study populations unbalanced in terms of baseline prognostic factors. This restriction was not present in the ipilimumab phase III trial.<sup>43</sup>

#### *Anti-CTLA-4 antibody in adjuvant therapy*

In a phase II trial, 75 patients with resected stage IIIC/IV melanoma were treated with ipilimumab to assess the safety and feasibility of this drug in an adjuvant setting.<sup>44</sup> Improved outcomes in terms of relapse-free survival and overall survival were reported compared with historical data; irAEs were generally reversible and appeared to be associated with improved relapse-free survival.<sup>44</sup> The European Organisation for Research and Treatment of Cancer (EORTC) melanoma group is conducting a phase III trial (EORTC 18071) where resected high-risk patients are randomized to ipilimumab treatment (dose 10 mg/kg every 3 wk for 4 cycles, then every 12 wk for a total of 3 y treatment) or to placebo in order to determine whether adjuvant ipilimumab can prevent disease recurrence. Recruiting has been completed and the results are pending.<sup>35</sup> In addition, a cooperative group trial is currently open to compare adjuvant ipilimumab to high-dose interferon  $\alpha$ -2b in treating patients with high-risk stage III or stage IV melanoma (ECOG 1609).<sup>35</sup> Given the minimal impact



**Figure 4.** T cell and cancer cell interaction.

of interferon in reducing disease recurrence, this study could provide additional evidence if immunotherapies have the potential to positively influence the immune system to eradicate minimal to microscopic residual disease resulting in prolonged OS.

#### Anti-PD-1 and PDL-1

Programmed death-1 (PD-1) receptor, also known as CD279, is a protein receptor that is inducibly expressed on CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells, natural killer T cells, and B cells. PD-1, interacting with programmed death ligand 1 (PDL-1) and PDL-2 negatively regulates immune responses.<sup>45-47</sup> PD-1 expression usually occurs on T cells during long-term antigen expression and induces T-cell exhaustion. The interaction between tumor cells expressing PD-L1 and T cells expressing PD-1 prevents an effective immune response by inactivating T-cells (Fig. 4).

Monoclonal antibodies such as BMS-936558 (nivolumab) and MK-3475 block this immune suppressive interaction of PD-1 and PDL-1. Nivolumab is a human monoclonal antibody targeting PD-1. A phase I study was conducted to test the efficacy and safety of a range of doses of nivolumab in patients with different cancers including 104 melanoma patients. The recommended phase II dose determined for nivolumab was 3 mg/kg IV every 2 wk, and the incidence of irAEs, especially grade 3–4, was lower for patients taking nivolumab compared with the historical data in ipilimumab-treated patients (14% vs 25.2%). Among patients with melanoma treated at the recommended phase II dose of 3 mg/kg, a response was noted in 7 out of 17 patients (41%).<sup>48</sup> Therefore, PD interference seems to display higher response rates and a beneficial side effects profile compared with CTLA-4 inhibition. A clinical trial is currently recruiting patients in order to directly compare efficacy and safety of nivolumab and ipilimumab (NCT01844505).<sup>35</sup>

More recently, a study that combined nivolumab and ipilimumab was completed in patients with advanced melanoma.<sup>49</sup> The concurrent administration of the two drugs every 3 wk, for 4 doses, was compared with a sequenced regimen where patients previously treated with ipilimumab received nivolumab every 2 wk for up to 48 doses. A higher objective-response rate was observed with the concurrent treatment regimen (40% vs 20%). However, grade 3 or 4 side effects occurred in 53% of the patients in the concurrent regimen and in 18% of patients in the sequenced-regimen group. The recommended phase II dosage for this regimen was nivolumab 1 mg/kg with ipilimumab 3 mg/kg.<sup>49</sup>

A second antibody targeting PD-1, MK-3475, also showed promising results with response rates as high as 52%. Among the 57 patients treated with 10 mg/kg IV every 2 wk, 91% experienced irAE but only 23% were grade  $\geq 3$ .<sup>50</sup> Two more trials have recently been launched to examine the effects of MK-3475 compared with chemotherapy (NCT01704287) and MK-3475 compared with ipilimumab (NCT01866319).<sup>35</sup> Preliminary data from trials with anti-PDL1 drugs (BMS-936559, MPDL3280A) do not seem to as promising as MK-3475 or nivolumab.<sup>51,52</sup>

#### Other modulators of immune regulatory checkpoints

##### CD40 agonist

CD40 is a co-stimulatory molecule expressed on dendritic cells, B cells, and monocytes (Fig. 3). Its ligand, CD40L, is expressed on CD4<sup>+</sup> T cells. The interaction of CD40–CD40L triggers T-cell activation. CD-870873 is a monoclonal antibody targeting CD40. In a phase I study, the administration of CD-870873 was associated with the induction of melanoma antigen-specific T cells, which is believed to be the mechanism of action leading to partial responses which were observed in



27% of patients with metastatic melanoma. The most common adverse event was cytokine release syndrome (grade 1 to 2) which included chills, rigors, and fever.<sup>53</sup> Preclinical data suggest a synergy between chemotherapy and CD40 agonists.<sup>54</sup> In a phase I 32 patients affected by advanced solid tumors were treated with a combination of CP-870893, paclitaxel and carboplatin. Out of them, 25 patients were affected by metastatic melanoma. Six of the 30 evaluable patients (20%) had a partial response and half of them were melanoma patients.<sup>55</sup> An ongoing trial is recruiting patients with stage IV melanoma in order to assess the most effective dose of CP-870893 in combination with tremelimumab and the related side effects (NCT01103635).<sup>35</sup>

#### *CD137 agonist*

CD137 is an inducible T-lymphocyte surface molecule of the tumor necrosis factor (TNF) receptor superfamily. Its ligand, CD137L, can be expressed by most immune and many non-immune cells as a transmembrane protein (Fig. 3). CD137 signaling enhances T-lymphocyte proliferation and T-helper-1 cytokine production, protecting CD8<sup>+</sup> T-lymphocytes from apoptosis. A phase I–II study of BMS663513, a human monoclonal antibody agonist of CD137, showed clinical activity with partial remission and stable disease in a subset of patients. However, a high incidence of grade 4 hepatitis was reported and the study had to be discontinued.<sup>56</sup>

#### *Agonistic antibodies targeting OX40*

OX40 and its ligand, OX40L (Fig. 3), are key TNF members that augment T-cell expansion, cytokine production, and cell survival. OX40 is a co-stimulatory molecule expressed transiently on the surface of T cells. Agonistic antibodies targeting OX40 have been shown to have antitumor activity.<sup>57</sup> A phase I with a proof-of-concept phase II trial was initiated to study in patients with unresectable or metastatic melanoma the combination of OX40 and ipilimumab, but the phase II trial to assess the immune system response to treatment with OX40 antibodies in monotherapy was withdrawn prior to enrollment due to changes in the development plan for the OX40 antibody (NCT01416844).<sup>35</sup>

#### *Anti-LAG-3 antibody*

Lymphocyte activation gene-3 (LAG-3) is a transmembrane protein that binds MHC class II molecules, enhances regulatory T-cell activity, and negatively regulates cellular proliferation, activation, and homeostasis of T cells (Fig. 3).<sup>58</sup> Further studies revealed a synergy between PD-1 and LAG-3 pathways to induce tolerance to self and tumor antigens.<sup>59</sup> Therefore, the dual blockade of these molecules might be a promising combinatorial strategy for cancer treatment. Moreover, melanoma cells often express MHC class II molecules, and the LAG-3-MHC II interaction protects tumor cells from apoptosis.<sup>59</sup> Targeting LAG-3 could serve as a bidirectional way to prevent tumor immune escape; however, the effects on autoimmunity are yet to be explored. A clinical trial to assess safety of anti-LAG-3 with or without anti-PD-1 in patients with solid tumors is currently recruiting patients (NCT01968109).<sup>35</sup>

#### *Anti-TIM-3 antibody*

T-cell immunoglobulin mucin-3 (TIM-3) is another inhibitory receptor expressed on a subset of tumor reactive T cells. Blockage of TIM-3 has been shown to improve the function

of anti-tumor T cells in different experimental models (Fig. 3). These findings provide the rationale for the clinical development of agents targeting these molecules. Interestingly, TIM-3 is frequently co-expressed with PD-1, and TIM-3/PD-1 expressing cells represented the majority of tumor-infiltrating T cells.<sup>60</sup> Combined targeting of the TIM-3 and PD-1 pathway will be an interesting future treatment approach, but to date clinical trials are not on going yet.

## **Biomarkers for Immunotherapy**

Several immunomodulatory strategies and combinatory approaches involving target inhibitors, immunotherapy, chemotherapy, surgery, and radiation have showed promising results in several clinical trials. However, a significant fraction of patients does not respond to treatment. The development of robust selection criteria and discovery of biomarkers predicting a response to certain treatment strategies is needed to improve response rates, disease-free survival, and OS. Selecting for the right patients will limit the risk of potentially severe irAEs to patients that are most likely to respond, thus greatly improving patient care.

#### **Interleukin-2**

Patients with limited subcutaneous and/or cutaneous metastases respond significantly better to IL-2 treatment than patients with other sites of disease.<sup>61</sup> Although high levels of serum VEGF, fibronectin,<sup>62</sup> and C-reactive protein<sup>63</sup> are not established as predictive biomarkers, they have been correlated with a lack of clinical response. A recent report<sup>7</sup> is suggestive of higher clinical responses to HD IL-2 in patients with NRAS mutations (47% responses in NRAS mutant melanoma patients vs 19% in wild type,  $P = 0.04$ ). To date, the majority of predictors for a response to IL-2 treatment have been post-treatment variables, such as magnitude of rebound lymphocytosis, treatment-induced thrombocytopenia, development of autoimmune thyroiditis and vitiligo and decrease in absolute peripheral T regulatory cell count.<sup>64</sup>

#### **Adoptive cell therapy**

Retrospective analyses have revealed several factors associated with response in ACT trials. The persistence of transfused cells in circulation after 1 mo,<sup>65–67</sup> the telomere length of infused cells<sup>68</sup> and the elevation of plasmatic levels of cytokines (such as IL-7 and IL-15<sup>69</sup>) were found to correlate with the response to ACT.

#### **Immune-checkpoint regulatory molecules**

Several large studies have reported increased efficacy of anti-CTLA-4 in patients who experience irAEs with responses in 26% of patients with irAE, compared with 2% in patients without.<sup>70–72</sup> There was also a “severity response effect” with slightly better response rates for patient grade 3–4 adverse reactions compared with patients with grade 1–2.<sup>70</sup> Several recent studies have shown that an absolute lymphocyte count (ALC) > 1000/μL with an increase in the ALC after 2 ipilimumab treatments correlates with clinical benefit (complete and partial response or stable disease 24 wk after beginning of the treatment) and OS.<sup>72,73</sup> In addition, increased expression of inducible costimulator molecule (ICOS) on T cells and a neutrophil/lymphocytes ratio below the median may represent an early marker of response.<sup>3</sup>

Other approaches trying to predict the response to immunotherapy focus on the tumor microenvironment. A recent study observed improved prognosis among patients with metastatic melanoma when a 12-chemokine gene expression signature (CCL2, CCL3, CCL4, CCL5, CCL8, CCL18, CCL19, CCL21, CXCL9, CXCL10, CXCL11, and CXCL13) was detected in the tumor microenvironment. This chemokine expression profile can be used to predict the likelihood that patients will respond favorably to immunotherapy.<sup>74</sup> A new concept known as immunoprofiling uses the genetically determined or tumor-induced immune response as a predictive factor for the response to immunotherapy.<sup>75</sup> Immunohistochemical analysis of pre- and post-treatment tumor biopsies from patients treated with ipilimumab has shown, that the tumor microenvironment may predict clinical activity. Baseline high expression of FoxP3 and indoleamine 2,3-dioxygenase (IDO), and a high count of tumor-infiltrating lymphocytes at baseline are related to a higher clinical activity of ipilimumab.<sup>76</sup> These biomarkers could help to develop an

immunoprofiling panel to select patients that are more likely to respond to anti-CTLA-4 therapy.<sup>75</sup>

Different studies suggest a relationship between PD-L1 expression on tumor cells and objective response to both anti-PD-1 antibodies<sup>48,77</sup> and anti-PD-L1 antibodies.<sup>52</sup> This also suggests that other immune checkpoint regulatory molecules such as anti-OX40, anti-TIM3 among others, may be useful to predict efficacy of therapeutics.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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